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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/82, 15/29, C07K 13/00, C12N 5/10, A01H 5/00

A1

(11) International Publication Number: WO 94/16088

(43) International Publication Date: 21 July 1994 (21.07.94)

(21) International Application Number: PCT/NL94/00011

(22) International Filing Date: 14 January 1994 (14.01.94)

(30) Priority Data:
9300078 15 January 1993 (15.01.93) NL

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(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
In English translation (filed in Dutch).

(54) Title: ANTI-NUTRITIONAL PROTEIN

(57) Abstract

An anti-nutritional protein such as a legume lectine which is mutated by replacing at least one amino acid of the natural protein sequence by a different amino acid in at least one surface loop of the protein, said surface loop containing one or more amino acids forming part of the ligand-binding site, so that the mutant protein has a reduced anti-nutritional effect. Also disclosed is a process for preparing the mutant protein, as well as a DNA-sequence and an expression vector used therein. Further, plant cells, plants and plant parts are disclosed wherein the mutant protein is expressed.

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Anti-nutritional protein

This invention relates to anti-nutritional proteins. In the feeding industry it is known that specific fodder components, such as seeds of leguminous plants, e.g. pea (Pisum sativum) and bean (Phaseolus vulgaris), cause problems. These components contain anti-nutritional proteins, also called anti-nutritional factors (ANF), such as lectins, trypsine inhibitors, haemagglutinins and tannins. These ANFs may bind to gut epithelial cells of animals such as pigs feeding on meal from the above-mentioned raw fodder components, thereby causing damage to the intestine (Hendriks et al., 1987; Huisman et al., 1990 (a); Huisman et al.; 1990 (b); Pusztai, 1989). In order to eliminate this adverse activity of ANFs, legume seeds have to be heat-treated for a considerable period of time. Apparently, ANFs are toxic in their native state and toxicity is lost upon irreversible denaturation. A standard heat treatment is toasting, that is steam heating for 40 min. at 104°C and 19% moisture (Huisman et al., 1990 (b). Toasting is energy-consuming and reduces the nutritional value of the seed and seed meal, making the use of legume seed meal as fodder for pigs economically unfavourable.

The object of this invention is to provide ANFs having a reduced or completely lost anti-nutritional activity in warmblooded animals and humans. Such ANFs need to be subjected to a less extreme heat treatment or even need no heat treatment at all to function as fodder.

Now it has been found that ANFs having a reduced anti-nutritional effect can be provided, which means that they retain their activity in the plant at ambient temperature, but lose their activity at the elevated temperatures in the intestine of cattle or pigs. Such ANFs retain their desired acitivity in the plant, but have a reduced or completely lost anti-nutritional activity in humans and domestic animals. Seeds containing such ANF may be fed to cattle without prior heat treatment or with a less extreme heat treatment than is used according to the present method.

Accordingly this invention provides an anti-nutritional protein which is mutated by replacing at least one amino acid of the natural protein sequence by a different amino acid in at least one surface loop of the protein, said surface loop containing one or more amino acids forming part of the ligand-binding site, so that the mutant protein has a reduced anti-nutritional effect.

Hereinafter the invention will be further illustrated and described more detailed while referring to legume lectins as example of anti-nutritional proteins.

Lectins of leguminous plants are carbohydrate-binding proteins present in high amounts in seeds and in lower amounts in roots (Díaz et al., 1990). In vivo, root lectins are involved in the host-specific symbiosis with Rhizobium bacteria (Díaz et al., 1989). The function of lectin in seeds is not known, but it has been hypothesized

that it can protect seeds and plants against herbivorous animals, including insects (Boulter et al., 1990; Bourne et al., 1990; Chrispeels et al., 1991; Pusztai, 1989).

Lectins of leguminous plants are among the best studied carbohydrate-binding proteins (Sharon et al., 1989); Sharon et al., 1990). The nucleotide sequences and the structures of a number of legume lectins is known (Becker et al., 1975; Einspahr et al., 1988; Einspahr et al., 1986; Reeke et al., 1986; Shaanan et al., 1991; Sharon et al, 1990). Thus, the lectin of the pea, Pisum sativum L., (PSL) is a so-called ß-barrel protein (Richardson et al., 1987) and is organised as a dimer, like the other lectins from the Viciae tribe of the Leguminosae family (Sharon et al., 1990). Both monomers are post-translationally processed into a small α- and a larger ß-chain. The mature protein therefore has a $\alpha_2ß_2$ configuration (Higgins et al., 1983). The overall three-dimensional structure of monomers of other legume lectins is very similar to that of the PSL monomer, in spite of differences in post-translational processing and multimerisation. The polypeptide chain of the native lectins is twisted in anti-parallel ß-sheet-structures forming the framework of the molecule. These ß-sheets are linked with each other through larger and smaller surface loops. The metal- and carbohydrate-binding sites are contained on these surface loops. (Einspahr et al., 1986; Einspahr et al., 1988).

Einspahr et al., 1986 present the structure of PSL in which the following surface loops are present, indicated with the amino acid numbers: (ß)9-17, 28-39, 47-62, 77-81, 87-115, 122-136, 142-147, 151-160, 167-172; (α) 195-206, 215-222. The position and structure of the surface loops of other legume lectins are also known.

As has been mentioned above according to the invention the lectin is mutated in at least one surface loop, said surface loop containing one or more amino acids forming part of the monosaccharide-binding site. For many lectins details of the monosaccharide-binding site are known, particularly which amino acids are involved in the binding of monosaccharides. It appears that the mono-saccharides bind to all lectins at corresponding sites and in a similar manner (Einspahr et al., 1986; Bourne et al., 1990 (a) and (b)).

It is preferred that in the mutant lectin an amino acid is replaced in a portion of the surface loop containing a conserved box of hydrophobic amino acids.

As mentioned above, the three-dimensional structures of the lectins of various leguminous plants are substantially identical. The degree of conservation is therefore high. This is shown in e.g. Sharon et al., 1990.

It is also preferred that in the mutant lectin an amino acid having a large hydrophobic side chain is replaced by a different amino acid.

Amino acids having a large hydrophobic side chain are valine, leucine, isoleucine, phenylalanine, tryptophan and methionine. Replacing such amino acids by amino acids having a smaller side chain greatly affects the stability of the lectin molecule. A particularly suitable replacing amino acid having a small side chain is alanine.

According to a specific embodiment an amino acid is replaced in the surface loop consisting of the amino acids 87-115 of the ß-chain of pea lectin or the corresponding amino acids of lectins of other leguminous plants.

Said surface loop is described for pea lecin by Einspahr et al., 1988. Fig. 1A and Table 1 show this loop. The -NH group of glycine-99 forms part of the monosaccharide-binding site (Bourne et al., 1990 (a)). The loop is twisted, and the amino acids at the position of this twist are conserved in all legume lectins (amino acids 101-104 in PSL; conserved box). Table 1 shows a comparison of the primary sequences of this surface loop of 14 different legume lectins (Sharon et al., 1990). The numbering is that of the PSL-sequence. Considering the great similarity of structure between the different lectins, also with respect to this surface loop, and the high degree of conservation of amino acids, the results obtained with PSL may be extrapolated to the other legume lectins.

The above-mentioned surface loop is present in all legume lectins, including jackbean lectin, Canavalia ensiformis (Concanavalin A), but it is discontinuous due to a circular permutation (Fig. 1B). In spite of this processing, the overall configuration of the loop including the conserved box is the same as in PSL.

It is particularly preferred to replace in the above-mentioned surface loop an amino acid of the conserved box, amino acids 101-104 of the ß-chain of PSL or the corresponding amino acids of lectins of other leguminous plants.

When in particular valine-103 of PSL is replaced by alanine a mutant lectin V103A is obtained having the following properties:

- In heamagglutination assays at room temperature no differences in properties between native (wild type, wt) PSL and PSL V103A could be observed. From this it can be concluded that the in vivo properties of PSL V103A have not been changed.

Table 1

	87												100																			115
PSL	I	A	P	V	D	T	K		P	Q	T		G	G	G	Y		L	G	V	F		N	S	A	E	Y	D	K	T	T	Q	T
LOL	I	A	P	V	D	T	K		P	Q	T		G	G	G	Y		L	G	V	F		N	S	K	D	Y	D	K	T	S	Q	T
LCL	I	A	P	V	D	T	K		P	Q	T		G	G	G	Y		L	G	V	F	Y	N	G	K	E	Y	D	K	T	S	Q	T
VFL	I	A	P	V	D	T	K		P	Q	T		G	G	G	Y		L	G	V	F		N	G	K	D	Y	D	K	T	A	Q	T
SBA	L	A	P	I	D	T	K		P	Q	T	H		A	G	Y		L	G	L	F		N	E	N	E			S	G	D	Q	V
DBA	L	V	P	V	G	S	E		P	R	R	N		·	G	Y		L	G	V	F	D	S	D	V	Y	N	N	S	A	G	Q	T
LTA	L	A	P	V	G	T	E	I	P	D	D	S	T	G	G	F		L	G	I	F	D	G	S			N	G	F	N	Q	F
PHA-L	L	V	P	V	G	S	Q		P	K	D	K		G	G	F		L	G	L	F	D	G	S			N	S	N	F	H	T
PHA-E	L	L	P	V	G	S	Q		P	K	D	K		G	G	L		L	G	L	F	N	N	Y	K		Y	D	S	N	A	H	T
SL	L	A	P	T	D	T	Q		P	K	S		G	G	G	Y		L	G	I	F		K	D	A	E	S	N	E	T			V
LBL	L	V	P	V	D	S	Q		P	K	K	K			G	R	L	L	G	L	F	N	K	S		E	N	D	I	N	A	L	T
DL	I	A	N	T	D	T	S	I	P	S	G	S		G	G	R	L	L	G	L	F	P		D	A	N	A	D		T			I
CONA	I	S	N	I	D	S	S	I	P	S	G	S	T		G	R	L	L	G	L	F	P		D	A	N	A	D		T			I
ECORL	M	G	P	T	K	S	K		P	A	Q		G	Y	G	Y		L	G	I	F	F	N	S	K	Q		D	N	S	Y	Q	T

Abbreviations used:

PSL = Pisum sativum lectin
LOL = Lathyrus ochrus lectin
LCL = Lens culinaris lectin
VFL = Vicia faba lectin (favin)
SBA = Glycine max (soybean) lectin
DBA = Dolichos biflorus lectin
LTA = Lotus tetragonolobus lectin
PHA = Phaseolus vulgaris lectin (phytohaemagglutinin)
SL = Onobrychis vicifolia (sanfoin) lectin
LBL = Phaseolus limensis (lima bean) lectin
DL = Dioclea grandiflora lectin
CONA = Canavalia ensiformis lectin (Concanavalin A)
ECORL = Erythrina corallodendron lectin

- After incubation at 70°C the agglutination activity was determined. Wt PSL lost its activity after incubation for 20-25 min., whereas PSL V103A was inactivated after incubation for 5 min. In both lectins, inactivation by incubation at 70°C appeared to be irreversible.

- In another assay it appeared that PSL V103A was irreversibly inactivated at 70°C, whereas wt-PSL lost its activity at 80°C.

- As most important result it was found that PSL V103A remains active at 28°C, but that the activity is lost at 37°C. Wt-PSL, on the contrary, is still completely active at 45°C and activity is lost at 55°C. Inactivation at these temperatures is reversible; upon cooling the activity can be restored.

Considering the high degree of conservation it may be expected that replacing the amino acid corresponding with valine-103 of PSL of lectins of other leguminous plants by alanine will give the same results.

This invention further provides a process for preparing the above-described mutant proteins having a reduced anti-nutritional effect, in which process in a protein encoding expression vector the DNA-sequence is changed by site-directed mutagenesis such that during expression in a suitable host cell the mutant protein is formed. This process will be described in detail in the part Materials and methods. Site-directed mutagenesis is a method known per se for the specific mutation of proteins.

This invention also provides a DNA-sequence comprising a sequence encoding a mutant protein as described above.

In the specific case of PSL V103A the encoding sequence equals the encoding sequence for wt-PSL, with the exception that the codon for valine-103, GTT, has been changed into GCT (coding for alanine).

This invention also provides an expression vector comprising the mutant protein encoding sequence and a suitable promoter sequence. The part Materials and methods will present examples of promoter and expression vector.

This invention provides also a plant cell comprising the above-mentioned DNA-sequence containing the promoter sequence and the mutant protein encoding sequence, which sequence is expressed in the plant cell forming the mutant protein having a reduced anti-nutritional effect.

The plant cell is particularly a leguminous cell. In this cell the gene for the mutant protein is incorporated and it has been secured that the wt-protein gene originally present is not expressed, by for example the anti-sense approach, see e.g. van der Krol et al., 1988. The plant cell may also be a cell of a plant in which the protein in question does not exist by nature. EP-A-0,351,924 describes transgenic plants such as tobacco or potatoe containing a PSL gene and wherein the lectin is expressed too. Instead of the wt-PSL gene the gene of the present invention could be used herein.

Finally this invention relates to plants regenerated from the above-mentioned plant cell, as well as to plant parts, particularly the seeds thereof. For the regeneration of pea plants reference can be made to Schroeder et al..

The invention will now be illustrated by the following example and with reference to the drawings, in which:

Fig. 1 shows the three-dimensional structures of the monomers of PSL (A) and CON A (B). The surface loop of both molecules is indicated with a thick line. Arrows indicate the processing sites involved in the circular permutation of CON A. An asterisk indicates the location of the sugar-binding site in both molecules (Becker et al., 1975; Einspahr et al., 1986);

Fig. 2 shows the position of Val-103 and Phe-104 in detail. The C-α-tracing of the surface loop (A) and the upper ß-pleated sheet (B) is shown. The approximate position of the sugar-binding site is indicated with an asterisk. Relevant amino acids are numbered and their side chains are indicated with thick lines;

Fig. 3 shows an immunoblot of crude E. coli extracts containing PSL.
Lane 1: Marker (pea seed lectin); lane 2: wt PSL; lane 3: PSL V103A; lane 4: PSL F104A; lane 5: PSL V103A/F104A. The polyclonal antiserum used does not react with the 6 kD α-subunit in lane 1;

Fig. 4 shows the heamagglutination assay with different concentrations of wt and mutant PSL;

Fig. 5 shows the heamagglutination-inhibition assay with wt and mutant PSL. The PSL concentration is 250 $\mu g.ml^{-1}$. Concentrations of glucose (GLC), mannose (MAN) and galactose (GAL) are indicated;

Fig. 6 shows the irreversible inactivation of wt and mutant PSL. Measurements were done in duplicate;

Fig. 7 shows temperature tracts. The PSL-concentration was 250 $\mu g.ml^{-1}$. The temperature was raised with 1°C. min^{-1} and aliquots were taken at the temperatures indicated;

Fig. 8 shows the heamagglutination assay at elevated temperatures. The PSL-concentration is 250 $\mu g.ml^{-1}$; in the last row no PSL is added;

Fig. 9 shows DSC-patterns of wt and mutant PSL. The peaks indicate the denaturation temperature (Tm), the area under the curves represents the total denaturation energy (ΔH). See also Table 2.

EXAMPLE

Materials and methods

Bacterial strains

E.coli strain DH5αF^+ (supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used for expression of wild-type and mutant lectin genes and for the production of PSL. Bacteria were grown in Luria Complete medium (Maniatis et al., 1982) (LC) at 37°C.

Cloning and site-directed mutagenesis of PSL

psl cDNA (Stubbs et al., 1986) was modified as described before (van Eijsden et al., 1992) by introduction of an extra EcoRI restriction site, resulting in an almost complete removal of the signal sequence, and was cloned into pUC 18 (Yannisch-Perron et al., 1985), to produce the expression vector pMP 2809. The N-terminus of produced lectin was determined using protein sequencing by automated Edman degradation on an Applied Biosystems 477A Protein Sequencer.

Mutations were introduced by use of the polymerase chain reaction (pcr) as described before (van Eijsden et al., 1992). For every mutation, an 89 bp EcoRV/BamHI fragment from pMP 2809 was amplified, using one mutagenic and one non-mutagenic primer. The codon for valine-103, GTT, was changed into GCT (coding for alanine) to produce V103A. The codon for phenylalanine-104, TTC, was changed into GCC (also coding for alanine), to produce F104A. The double mutant was made by combining both mutations in a single mutagenic primer. Total amplified DNA was sequenced according to the method of Sanger et al. (Sanger et al., 1977), using sequenase 2.0™ (USB, Cleveland, Ohio, USA). After checking the 89 bp fragment by means of DNA sequence analysis, the original EcoRV/-BamHI fragment from pMP 2809 was replaced by the fragments containing the mutations, yielding pMP 3203, pMP 3204, and pMP 3211 respectively.

Isolation of PSL from E.coli

Isolation of PSL from E.coli was performed as described before (Prasthofer et al., 1988; Van Eijsden et al., 1992), with some modifications: E.coli DH5αF$^+$ cells, harbouring pMP 2809 or one of its derivatives, were grown in 2L LC at 37°C, containing 100 µg/ml carbenicillin, and were induced at mid-exponential phase by adding IPTG (isopropyl-ß-D-thiogalactopyranosid; Boehringer, Mannheim) to the medium to a final concentration of 0.5 mM. After induction, the cells were grown for an additional 16 h at 37°C, harvested and washed in TBS (10 mM Tris-HCl, pH 6.8, containing 150 mM NaCl). All further steps were performed at 4°C, unless stated otherwise. The cells were resuspended in 25 ml TBS containing 0.5 mM PMSF (phenyl-methylsulphonylfluoride) and lysed in a French pressure cell (American Instrument Company, Silver Spring, MA, USA), at a pressure of 1500 psi. Inclusion bodies

containing PSL were collected by centrifugation for 30 min. at 15,000 rpm. The protein was denaturated overnight in TBSm (TBS containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$) in the presence of 7M guanidine-HCl. Membranes and remaining aggregates were removed by ultracentrifugation for 1 hr at 175,000 x G. The supernatant was quickly diluted 25-fold in TBSm containing 1.5 M urea at 0°C. The proteins were allowed to refold during at least 24 h, after which the solution was dialysed extensively against deionised H_2O and lyophilized. Finally, the lyophilized proteins were redissolved in TBSm and purified by affinity chromatography at room tempera-ture on Sephadex G-75 (Pharmacia, Uppsala, Sweden) in TBSm (Díaz et al., 1990).

SDS-PAGE of PSL fractions was performed with a 15% running gel according to Lugtenberg et al. (1975). After running, the gels were blotted onto PVDF membrane (Millipore, Bedford, MA, USA). Subsequently, immunochemical staining with the use of polyclonal anti-PSL antibodies was performed according to Díaz et al., (1990).

Haemagglutination assays

The haemagglutination assay used to test the sugar-binding ability and sugar-binding specificity of PSL was described before (Kijne et al., 1980). In order to test stability, a PSL solution of 250 $\mu g.ml^{-1}$ in TBSm was incubated at 70°C. Aliquots were taken at 5 min. intervals and placed on ice. Subsequently, these samples were tested in the haemagglutination assay for residual activity.

In another experiment, a PSL solution of 250 $\mu g.ml^{-1}$ in TBSm was submitted to a temperature tract using a Biozym thermal cycler (Wessex, Andover Hampshire, England). The temperature was raised with 1°C.min^{-1}, ranging from 20°C to

80°C. Aliquots were taken after every 10°C increase, placed on ice, and tested in the haemagglutination assay.

Finally, haemagglutination assays were performed at different temperatures (28°C, 37°C, 45°C and 55°C) instead of 20°C, to try to distinguish between reversible and irreversible inactivation.

Differential Scanning Calorimetry (DSC)

In order to determine the denaturation temperature (Tm) and -energy (ΔH) of PSL, a Mettler TA-300 DSC apparatus, coupled to a TC-10 detector, was used. Scanned temperature tracts were 5 to 100°C, and the scanning speed was 10°C.min^{-1}. The samples contained 80% (w/w) H_2O.

Results

Production of wild-type and mutant PSL in E.coli

Plasmid pMP 2809, containing psl cDNA, was expressed in E.coli. This resulted in the production of unprocessed PSL containing 5 extra amino acids at the N-terminus, i.e. three amino acids from the pUC 18 sequence and two from the original signal peptide. This molecule was designated wt PSL (Van Eijsden et al., 1992). The inventors could typically isolate 10-20 mg of affinity-purified wt PSL from a 2L E.coli culture. Introduction of the mutations V103A, F104A and V103A/F104A in pMP 2809 yielded plasmids pMP 3203, pMP 3204, and pMP 3211 respectively. The yield of PSL F104A was similar to that of wt PSL. However, PSL V103A was much more difficult to obtain, since only about 2 mg could be isolated from a 2L culture. Wt and mutant PSL monomers all have an apparent molecular weight of

about 28 kD as judged from SDS-PAGE (Fig.3), corresponding with that from unprocessed PSL from pea seeds. The double mutant, PSL V103A/F104A, was also produced in E.coli, but could not be isolated in an active form by affinity chromatography. The molecular weight of native wt and mutant PSL, as judged from gel filtration experiments, appeared to be about 55 kD. From this result it can be concluded that the introduced mutations did not affect PSL dimerisation.

Haemagglutination assays

Wt PSL, PSL V103A and PSL F104A all agglutinated a 2% suspension of human A^+ erythrocytes down to a minimum concentration of about 16 µg/ml (Fig.4). No significant differences in sugar-binding properties of the mutants could be observed: mannose inhibits haemagglutination at a minimal concentration of 3 mM, glucose inhibits haemagglutination at a minimal concentration of 12.5 mM, and galactose does not inhibit haemagglutination at a concentration as high as 100 mM (Fig.5).

After incubation of PSL for various periods at 70°C, residual agglutination activity was determined. Wt PSL and PSL F104A lost their activity after incubation for 20-25 min. at 70°C, whereas PSL V103A was inactivated after incubation for 5 min. at 70°C. In all cases, inactivation by incubation at 70°C appeared to be irreversible, since activity was not restored by subsequent incubation on ice for up to 30 min. (Fig. 6).

Wt PSL and PSL V103A were subjected to a temperature tract ranging from 20 to 80°C in a thermal cycler. PSL V103A irreversibly lost its activity at 70°C. Wt PSL lost its activity at 80°C in the same assay (Fig. 7).

By assaying haemagglutination at elevated temperatures, it could be demonstrated that PSL V103A remains active at 28°C, but that the activity of this mutant is lost at 37°C (Fig. 8). PSL F104A was still active at 37°C, but activity diminished at 45°C. Wt PSL was completely active at 45°C, but activity was lost at 55°C. Inactivation at these temperatures is reversible upon cooling, as is shown by the combined results from the experiments shown in Figures 7 and 8. Assaying haemagglutination at these temperatures did not have a visible effect on the erythrocytes used.

Differential Scanning Calorimetry (DSC)

The denaturation temperature (Tm) and denaturation energy (ΔH) of wt PSL and the two mutants were determined using DSC. The results are shown in Table 2. The DSC curves are shown in Fig. 9. Tm and ΔH of wt PSL isolated from pea seeds are not significantly different from the Tm and ΔH of wt PSL isolated from E.coli (data not shown). The Tm of PSL V103A is approximately 10°C lower than that of wt PSL, whereas ΔH is only about 55% of ΔH of wt PSL. PSL F104A does not show a significant difference in Tm or ΔH when compared with wt PSL. These results corroborate the results of the haemagglutination experiments.

Table 2

PSL	Tm (°C)	ΔH (kCal/mole)
wt	82.3 (± 0.7)	164 (± 6)
V103A	71.4 (± 1.0)	88 (± 4)
F104A	81.8 (± 0.5)	145 (±15)

The DSC patterns presented in Fig. 9 show single denaturation peaks in wt and both mutant lectins. However, the optimum of the pattern of V103A is shifted about 11°C and is at 71°C. This implies that the mutation in PSL V103A facilitates total denaturation of the protein. A local effect on the conformation of the surface loop could not be detected in this experiment.

Performing the different haemagglutination experiments described above, one can make a distinction between reversible and irreversible inactivation of PSL. Reversible inactivation of a PSL solution in TBSm occurs at temperatures between 28 and 55°C, and irreversible inactivation occurs between 60 and 80°C. PSL V103A is less stable than wt PSL or PSL F104A. PSL F104A is slightly less stable than wt PSL at 45°C, but at higher temperatures this difference is not found. Irreversible inactivation is likely to coincide with the complete denaturation of the protein, whereas reversible inactivation probably can be attributed to local conformational changes in the surface loop, either causing local denaturation of this loop or causing an overall decrease in sugar-binding affinity.

The refolding of denaturated PSL seems to require very specific circumstances. This phenomenon is especially encountered upon renaturation of PSL from E.coli inclusion bodies, and it would also explain why PSL V103A is more difficult to isolate than wild-type lectin, and why the double mutant, V103A/F104A, cannot be isolated in an active form at all.

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C L A I M S

1. An anti-nutritional protein which is mutated by replacing at least one amino acid of the natural protein sequence by a different amino acid in at least one surface loop of the protein, said surface loop containing one or more amino acids forming part of the ligand-binding site, so that the mutant protein has a reduced anti-nutritional effect.

2. A protein according to claim 1, **characterized in that** at least one amino acid is replaced in a portion of the surface loop containing a conserved box of hydrophobic amino acids.

3. A protein according to claim 1 or 2, **characterized in that** at least one amino acid having a large hydrophobic side chain is replaced by a different amino acid.

4. A protein according to claim 3, **characterized in that** the replacing amino acid is alanine.

5. A protein according to one of the claims 1 to 4, **characterized in that** said protein is a legume lectin.

6. A legume lectin according to claim 5, **characterized in that** the surface loop having at least one amino acid replaced consists of the amino acids 87-115 of the ß-chain of pea lectin or the corresponding amino acids of lectins of other leguminous plants.

7. A legume lectin according to claim 6, **characterized in that** at least one of the amino acids 101-104 of the ß-chain of pea lectin or the corresponding amino acids of lectins of other leguminous plants is replaced by a different amino acid.

8. A legume lectin according to claim 7, **characterized in that** valine-103 of the ß-chain of pea lectin or the corresponding amino acid of lectins of other leguminous plants is replaced by alanine.

9. A process for preparing an anti-nutritional protein according to one of the claims 1 to 8, **characterized in that** in a protein encoding expression vector the DNA-sequence is modified by site-directed mutagenesis such that during expression in a suitable host cell the mutant protein is formed.

10. A DNA-sequence comprising a sequence encoding a protein according to one of the claims 1 to 8.

11. An expression vector comprising a DNA-sequence as defined in claim 10 and a suitable promoter sequence.

12. A plant cell comprising a DNA-sequence according to claim 10, which is expressed in the plant cell forming the mutant protein having a reduced anti-nutritional effect.

13. A leguminous plant cell according to claim 12.

14. A plant regenerated from a plant cell as defined in claim 12 or 13, as well as plant parts, particularly seeds.

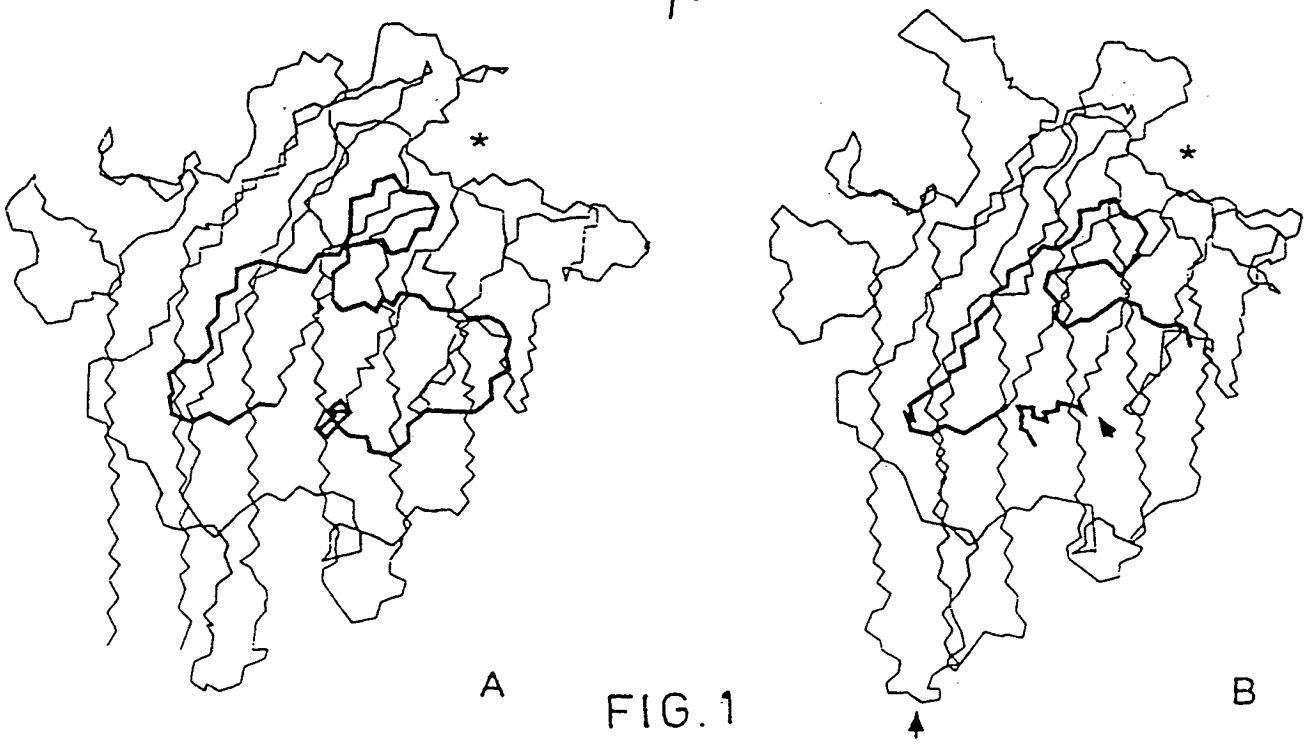

FIG. 1

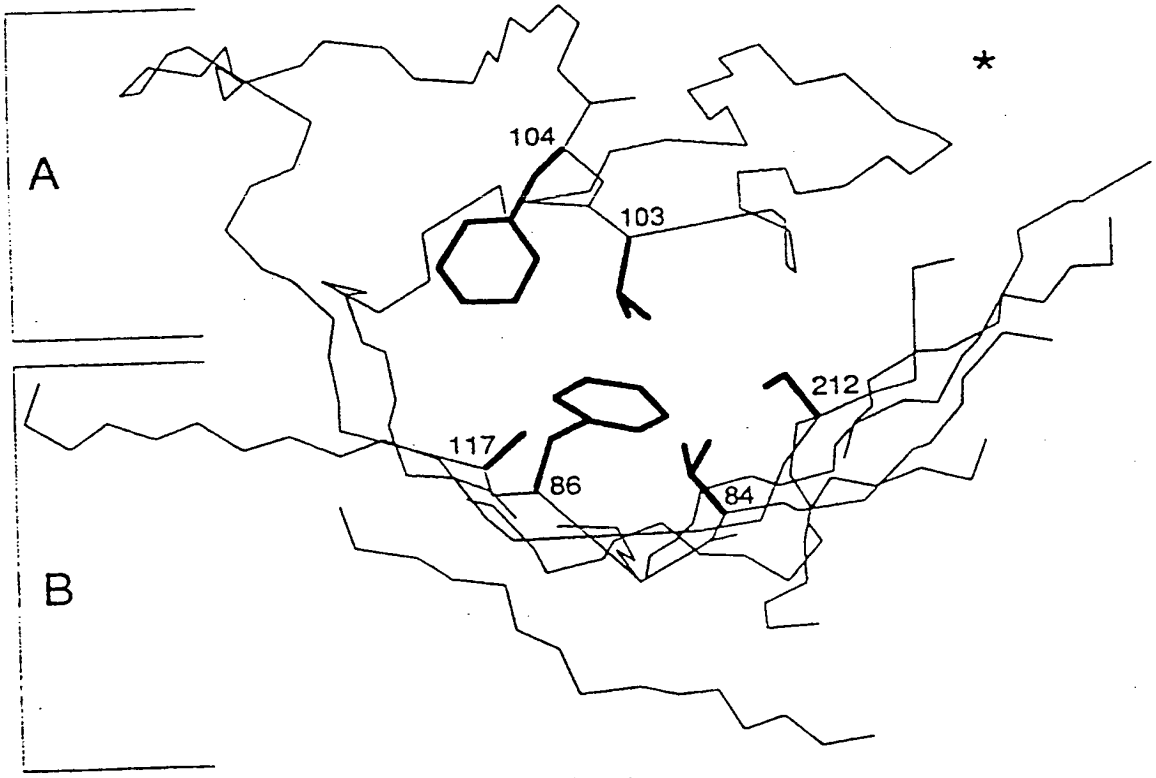

FIG. 2

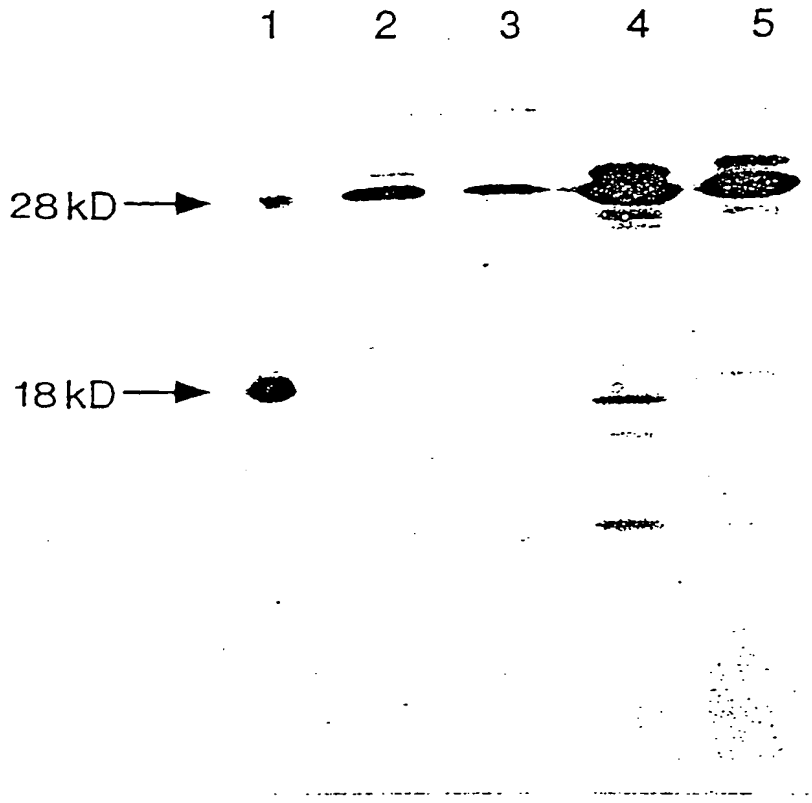

FIG. 3

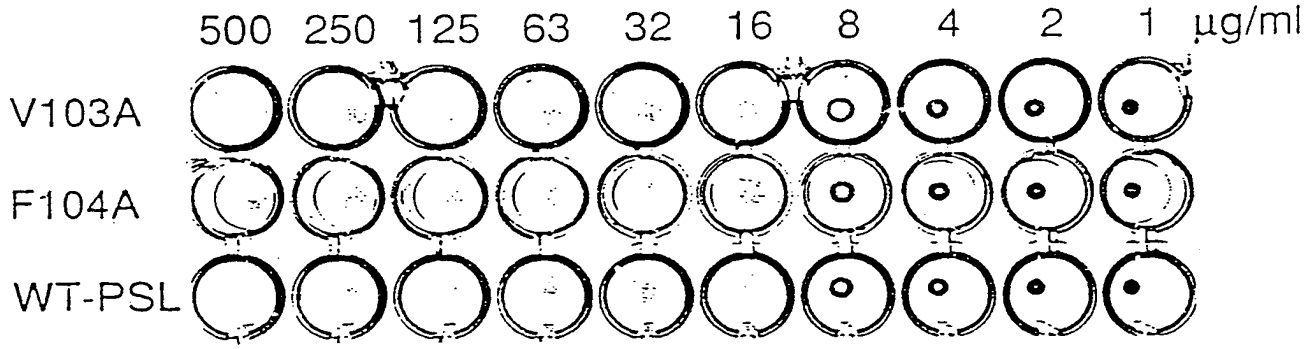

FIG. 4

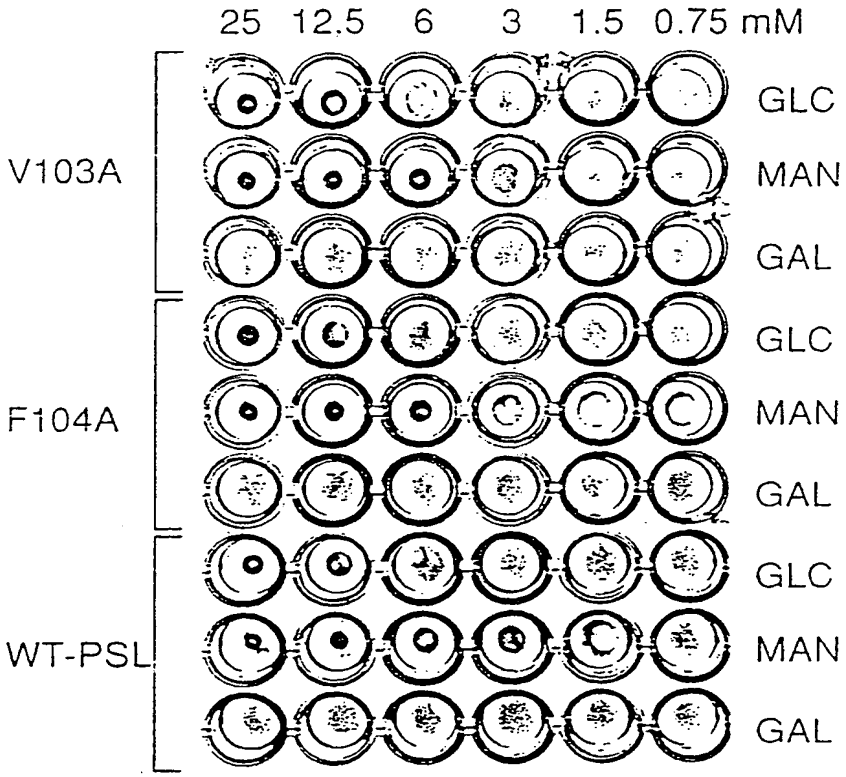

FIG. 5

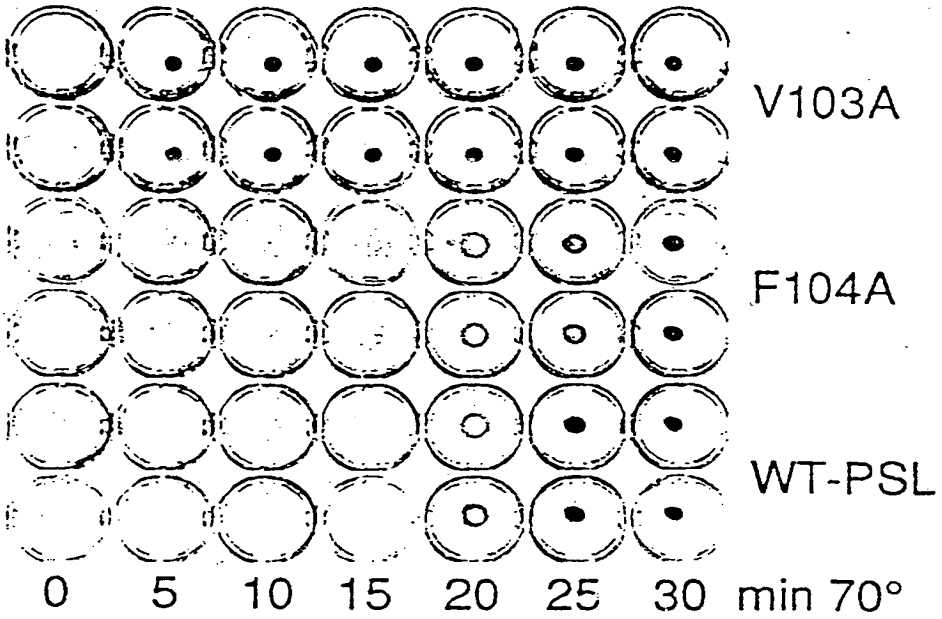

FIG.6

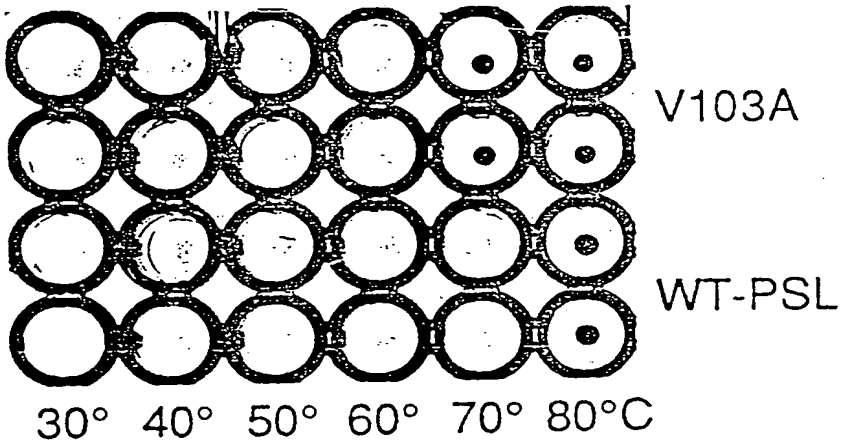

FIG.7

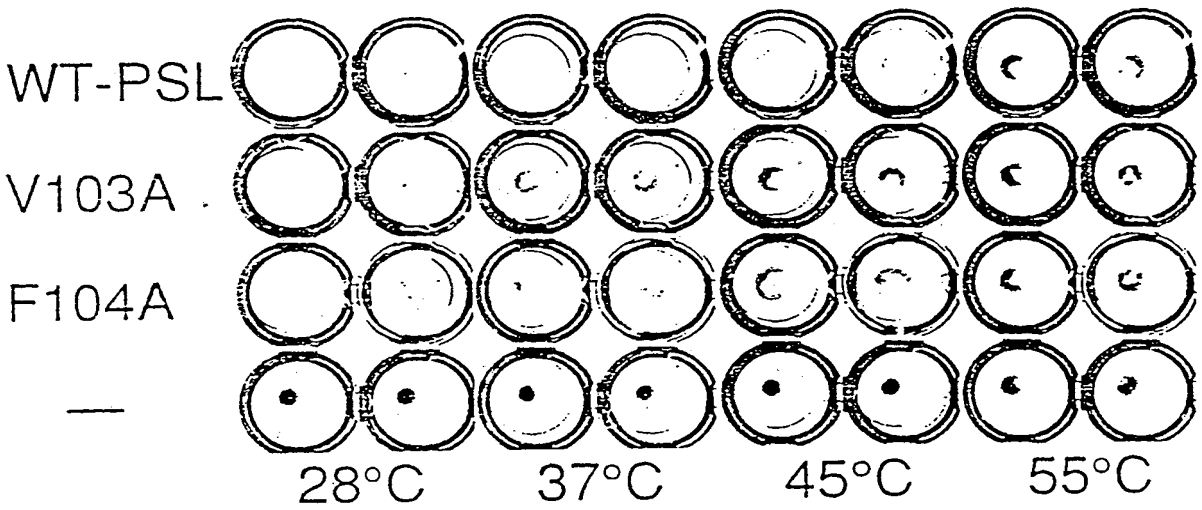

FIG. 8

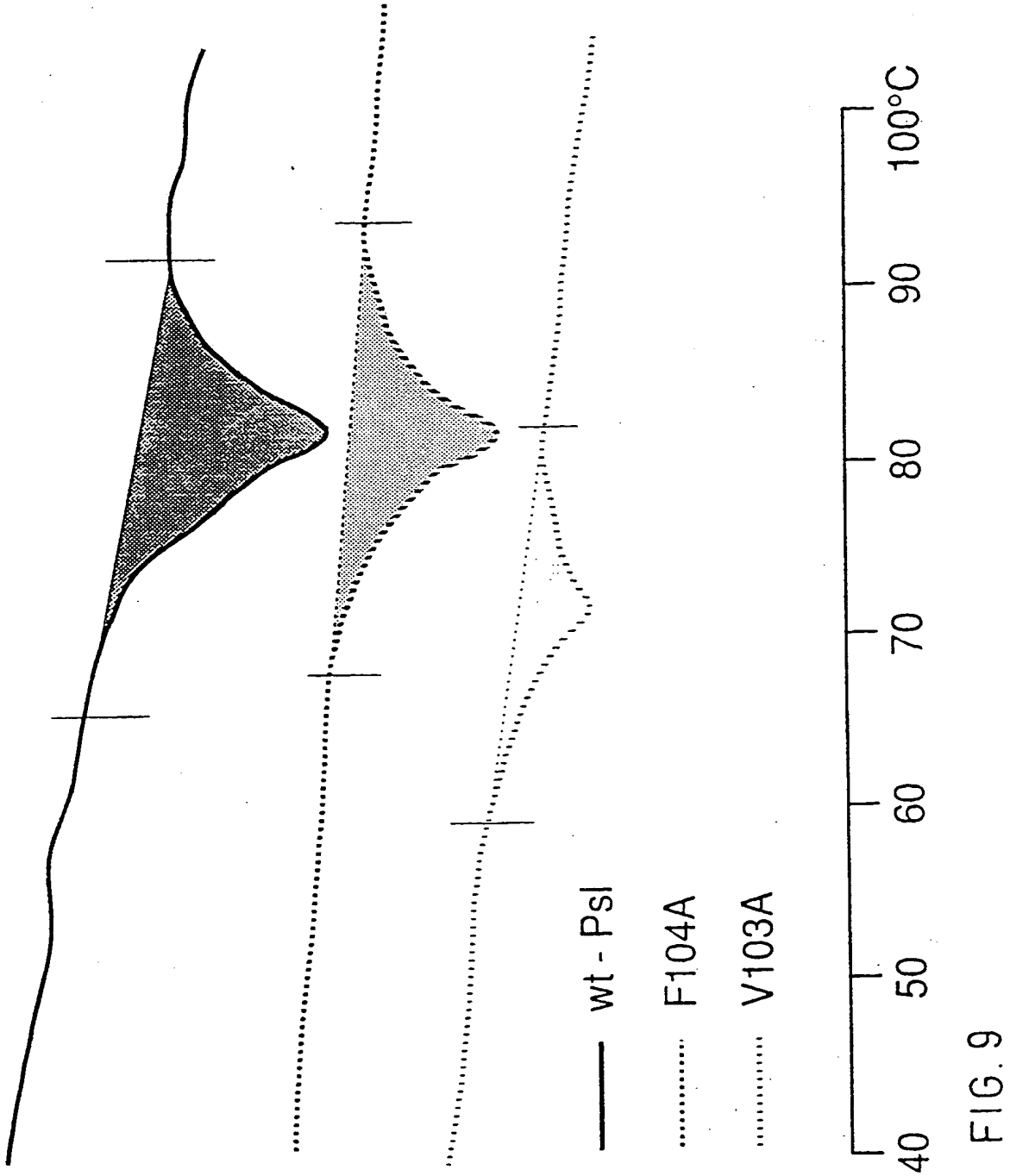

FIG. 9

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/NL 94/00011

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/82 C12N15/29 C07K13/00 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PLANT MOLECULAR BIOLOGY. vol. 22, no. 6 , September 1993 , DORDRECHT, THE NETHERLANDS. pages 1039 - 1046 HOEDEMAEKER, F.J., ET AL. 'Destabilization of pea lectin by substitution of a single amino acid in a surface loop' see the whole document --- -/--	1-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 May 1994	01 -06- 1994

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Inter ..al Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PLANT MOLECULAR BIOLOGY. vol. 20, no. 6 , 1992 , DORDRECHT, THE NETHERLANDS. pages 1049 - 1058 VAN EIJSDEN, R.R., ET AL. 'Mutational analysis of pea lectin substitution of Asn-125 for Asp in the monosaccharide-binding site eliminates mannose-glucose-binding activity' see the whole document ---	1-14
A	EP,A,0 174 166 (AGRIGENETICS) 12 March 1986 see page 27, line 26 - line 33; examples 4,5,8,9 ---	10-14
A	EP,A,0 351 924 (SHELL) 24 January 1990 see the whole document -----	10-14

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INTERNATIONAL SEARCH REPORT

...information on patent family members

Inter ...al Application No
PCT/NL 94/00011

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0174166	12-03-86	AU-A- 4693085	06-03-86
		JP-A- 61081793	25-04-86
EP-A-0351924	24-01-90	JP-A- 2107137	19-04-90